IDENTIFICATION OF LIGANDS FOR A RECEPTOR CAPABLE OF INTERNALIZING

The present invention relates to a screening method which can be used for identifying potential ligands for a receptor capable of internalizing.

The invention relates more particularly, but not exclusively, to the receptors belonging to the family of 7-transmembrane domain G protein-coupled receptors and also to those of the family of single-transmembrane domain tyrosine kinase receptors.

To date, approximately 800 7-transmembrane domain G protein-coupled receptors (GPCRs) have been cloned from various eukaryotic species. In humans, 240 GPCRs have been isolated. The endogenous ligand is known for only 140 of them, the other remaining 100, which constitute the orphan receptor pool, are still to be identified.

In parallel, several hundreds of novel medicinal products which act on GPCRs have been registered over the past twenty years.

Consequently, newly cloned orphan receptors are of great interest for pharmaceutical research since they may represent potential therapeutic targets. However, their development on a therapeutic level first involves isolating their endogenous ligand and, by the same token, elucidating their function.

Out of all of these G protein-coupled orphan receptors, only 5 endogenous ligands have to date been isolated, all belonging to families of novel peptides: orexins/hypocretins, nociceptin/orphanin, PrRP, prolactin-releasing peptide, apelin and the leucokinin-like peptide.

In order to identify them, the authors have, in all cases, screened purified tissue fractions on various tests.

For the choice of tests, they have taken advantage of the fact that, when an agonist binds to its receptor, a second messenger is formed which will produce, depending on the signaling pathway used by the receptor, various products and which will, in all cases, result in a change in intracellular pH.

In the case of nociceptin, the authors have measured cAMP accumulation resulting from the stimulation of adenylate cyclase.

In the case of the orexins, the authors have measured intracytoplasmic Ca2+ accumulation resulting from the stimulation of phospholipase C.

In the case of PrRP, the authors have measured arachidonic acid formation resulting from the stimulation of phospholipase A2.

In the case of apelin, they have measured extracellular medium acidification modifications using a "cytosensor".

However, this set of identification pathways is not totally satisfactory for the following reasons:

The approach for identification consisting in evaluating the production of second messengers requires knowledge of the signaling pathway of the receptor. In the presence of an orphan receptor, having no idea of the pathway used, it proves necessary to test all of the known signaling pathways in the hope that the receptor of interest is not coupled to a pathway which is still unknown. This therefore requires having a considerable amount of sample. This approach also

implies that the receptor of interest is coupled to a cascade of second messengers in the heterologous transfection systems in which they are expressed, which is not necessarily the case.

With regard to the approach consisting in measuring the acidification of the extracellular medium, subsequent to the production of protons by the activated cell, it comes up against the following problem: if libraries of peptides or purified fractions of tissue extracts are placed together with an orphan receptor expressed at the surface of a cell, a modification of extracellular pH is measured which is the result of the stimulation not only of the orphan receptor but also of all the endogenous receptors present. The ligand responsible for the activation of the orphan receptor cannot therefore be easily determined.

The object of the present invention is precisely to propose a novel method for detecting and/or identifying orphan receptor ligands, which proves to be more reliable than those mentioned above, which can be carried out on samples with small volumes and which can be exploited in the presence of other endogenous receptors and of a considerable number of ligands, which may be peptide or nonpeptide ligands, for these supplementary endogenous receptors.

The method developed in the context of the present invention takes advantage of the properties which seven-transmembrane domain G protein-coupled receptors or tyrosine kinase receptors have of internalizing in the cells which express them, under the action of agonist ligands. Internalization is a fairly universal phenomenon which affects a large number of receptors. This internalization can thus be visualized by conconfocal, optical or even electron microscopy, when the ligand is labeled with a fluorescent molecule or an epitope label. The ligand-receptor complexes follow

then a characteristic intracellular path, which has already been studied. For example, this technique of fluorescent or epitope labeling has already been advocated for monitoring the intracellular trafficking either:

- of a labeled ligand complexed with its respective receptor, said receptor undergoing internalization induced by its activation,

- of a protein involved in signal transduction, in this case labeled protein kinase C,

- or of a protein involved in the desensitization of a receptor after it has been activated, in this case labeled β-arestin 2.

However, the option consisting in labeling either a ligand or a protein involved in signal transduction or in the desensitization of a receptor after it has been activated, such as labeled β-arestin 2, is not completely reliable. An ambiguity may, in fact, remain concerning the identity of the receptor, the internalization of which was monitored. In addition, recent data suggest that different proteins may be involved in the mechanisms of internalization and of desensitization of diverse receptors. β-Arestin 2, in particular, does not appear to play a universal role.

For example, when the mobilization of EGFP-coupled β-arestin is monitored during the stimulation of the orphan receptor by its ligand, the receptor is phosphorylated and can then bind the β-arestin 2-GFP mobilized from the cytoplasm to the membrane. The receptor is then sequestered and internalized. Now, when an endogenous ligand for an orphan receptor overexpressed at the surface of eukaryotic cells is sought, the bringing into contact with a library of peptides or purified tissue fractions results in the

activation not only of the orphan receptor but also of all the endogenous G protein-coupled receptors. As a result of this, therefore, there is mobilization of the β-arestin 2-GFP not only by the orphan receptor but also by the endogenous receptors. This does not therefore make it possible to identify the ligand responsible for the activation of the orphan receptor.

Similarly, in the case of a library of peptides or purified tissue fractions containing the endogenous ligand being sought, if all the peptides are labeled homogeneously, it is not possible to identify the one being sought.

The advantage of the screening method claimed is precisely that it removes this uncertainty.

More precisely, the present invention relates to a method which can be used for detecting and/or identifying, in a library of peptide, pseudopeptide or nonpeptide compounds, a biological extract and/or a purified fraction of a tissue extract, a ligand for a receptor of interest which is capable of undergoing an internalization induced by the binding of said ligand, characterized in that it comprises at least the steps consisting in:

- expressing said receptor in a labeled form at the surface of a cell,

- placing said cell together with said library, the biological extract and/or a purified fraction of a tissue extract containing at least one peptide, pseudopeptide or nonpeptide compound likely to be a ligand for said receptor, under conditions sufficient to allow cellular internalization of said receptor-ligand complex and

- visualizing this internalization via the detection of the label attached to said receptor.

The present invention therefore involves the labeling of the orphan receptor for which potential agonists are in particular being sought.

The fact of labeling the orphan receptor is, in itself, clearly advantageous with regard to the detection techniques mentioned above. Specifically, this approach offers the possibility of directly visualizing the target studied. When the receptor is brought into contact with the endogenous ligand, it is the ligand-receptor-label complex which is internalized. There is no ambiguity concerning the identify of the receptor internalized.

In addition, in the method claimed, the cell constitutively overexpresses the labeled receptor. The labeling is intracellular, on the cytoplasmic tail of the receptor, and therefore has the advantage of not hindering the binding of the ligand.

With regard to the labels which are suitable for the invention, they may be either an autofluorescent protein or an epitope label which can be detected by immunohistochemistry.

The fluorescent proteins which can be used in the method claimed preferably belong to the family of the wild-type fluorescent protein GFP and mutants thereof (Ex: EGFP, EBFP and EYFP), Wang S. & Halzelrigg T. (1994) Nature, 369:400-403; Yang TT et al. (1996) Nucleic Acid Res, 24: 4592-4593; Heim R & Tsien RY (1994) Curr. Biol., 6:1, 178-182; Ormö M et al., (1996) Science, 273: 1392-1395.

By way of illustration of nonfluorescent labels which can also be used according to the invention, mention

may be made most particularly of hemagglutinin, polyhistidine, the myc and flag proteins and viral epitopes. For all these highly immunogenic compounds, commercially available high affinity selective antibodies already exist (ex: antimyc monoclonal antibodies, Clontech; anti-influenza hemagglutinin antibody, Boehringer; anti-vesicular somatitis virus antibody, Clontech; antipolyhistidine antibody, InVitrogen). The detection thereof involves recognition of the antigenic group by one of these antibodies (termed primary antibodies), followed by visualization of the primary antibody with a secondary antibody which comes from another species and which is labeled either with a fluorophore or with horseradish peroxidase which will subsequently be reacted with a suitable substrate.

This type of labeling is particularly advantageous since it enables a high sensitivity of measurement which results in the detection of a ligand concentration of about 10^{-8} M, i.e. 100 fmol in 10 μl. The internalization of the complex is visible by confocal microscopy or even optical microscopy when the receptor is highly expressed and if there is a sufficient concentration of the ligand (minimum of 10^{-8} M).

This internalization is preferably detected by confocal and/or optical microscopy.

Finally, advantageously, the other endogenous receptors, which are not labeled, and which present at the surface of the host cells, even if they are also internalized, are not visible and do not interfere with the measurement level. No background noise is therefore observed, which is particularly advantageous with regard to the high sensitivity of the method of measurement.

In general, the method claimed proves to have a sensitivity which is sufficient to enable the visualization of an internalization of a receptor-ligand conjugate in the fraction tested, notwithstanding the presence of other endogenous receptors and of a large number of peptide or nonpeptide ligands for these supplementary endogenous receptors.

Advantageously, the screening method claimed proves to be suitable for studying any receptor, on condition that the latter has the capacity to internalize.

According to a preferred method of the invention, the receptor of interest is coupled to a G protein.

The method claimed is particularly advantageous for characterizing ligands for receptors belonging to the family of 7-transmembrane domain G protein-coupled receptors, GPCRs, to the family of single-transmembrane domain tyrosine kinase receptors or to the family of cytokine receptors.

In the case of the GPCRs, the C-terminal tagging of the receptors has the following advantages:

- the post-translational maturation of the receptor is not modified,
- the binding of the agonists and of the antagonists, and also the intracellular signaling, are the same as with the native receptor,
- the internalization of the ligand-receptor complexes is not modified and
- the method for detecting the expression is rapid and can be carried out on live unfixed cells.

The receptor to be studied is coupled to a label and then expressed at the surface of a host cell. With regard to these two operations, namely the labeling and the expression of said receptor in a host cell, they are both, of course, within the competence of those skilled in the art.

In general, the following procedure is carried out:

The sequence of the coding region of the receptor of interest is inserted in frame into an expression vector, upstream or downstream of the coding sequence of the fluorescent protein (GFP, EGFP, EBFP, EYFP) or of an epitope label. These expression vectors (of the pGFP-N1 or pGFP-C1 type, N1 or C1 indicate the position of the receptor relative to the protein, either in the N-terminal position or in the C-terminal position) already contain the sequence of these labels. The cells are transfected using a conventional method (such as the liposome method, calcium phosphate), and then selected for their resistance to an antibiotic. In the case of the proteins of the GFP family, it is possible to sort the cells expressing the receptor coupled to the fluorescent protein, by flow cytometry.

With regard to the transformed cells, they are eukaryotic cells, such as for example monkey kidney epithelial cells: COS-7; hamster ovary epithelial cells: CHO; and human embryonic kidney cells: HEK 293.

In a variant of the method claimed, it is possible to envision expressing, at the surface of a cell, two or more different receptors labeled, respectively, with different labels. The two receptors are, of course, capable of internalizing. This option thus offers the possibility of screening, on the same sample and simultaneously, potential ligands for several receptors.

In accordance with the method claimed, the host cells are placed together with a potential ligand. To do this, said cells are therefore brought into contact with either a library of peptide, pseudopeptide or nonpeptide compounds, a biological extract or purified fractions of a tissue extract.

This bringing into contact is carried out under conditions which are sufficient to allow the cellular internalization of the receptor-label-ligand complex(es). These sufficient conditions are of course assessed by those skilled in the art, in terms of temperature, of duration and of concentration. It may also be necessary to carry out repeat experiments.

As a general rule, the internalization of ligand-receptor complexes in mammalian cells is optimal at 37°C. The mean halflife of the internalization process (time required for 50% of the surface receptors occupied to be internalized) is about 10 minutes. Thus, 20 to 40 minute exposures to the ligand are conventionally optimal for observing the internalized receptors; the optimal ligand concentrations are about 10 to 20 times the affinity of the ligand for its receptor (Kd).

The internalization of the ligand-receptor complex, which materializes as the movement of the label (fluorescent or immunohistochemical) from the membrane of the cell (the intensity of labeling of which decreases) toward the inside of the latter, in the form of vesicles, is monitored by confocal microscopy or, in the case of a high expression of this labeled receptor, by optical microscopy.

In the case of a receptor labeled with a fluorescent tag, the visualization takes place directly by observing the movement of the fluorescence.

When the receptor is labeled with a nonfluorescent antigen, the internalization is visualized by confocal microscopy, after fixing the cells and detecting the antigenic epitopes with secondary antibodies coupled to a fluorophore. Alternatively, the antigenic epitopes may be revealed with an enzymatic reaction or using a radioactive antibody, which, in both cases, materializes as the accumulation of opaque deposits visible both by photon microscopy and electron microscopy.

As mentioned above, the internalization may be visualized on ten or so cells and for a very small incubation volume, i.e. about one μl. These two qualities are particularly valuable when only a very small amount of sample is available.

Consequently, the method claimed proves to be most particularly advantageous for detecting and/or identifying the endogenous ligand(s) for an orphan receptor, or identifying novel agonists or antagonists for a known receptor, i.e. a receptor for which the endogenous ligand is known.

Since this method is based on the direct observation of a biological phenomenon which affects most GPCR or tyrosine kinase receptors, namely internalization, it proves to be particularly useful in searching for the ligands for orphan receptors, and more particularly for peptide receptors, using biological preparations such as tissue extracts.

It is also possible to envision using the method claimed with a labeled receptor which has already been identified and characterized, such as a "biosensor", which would make it possible to detect in a biological liquid the presence, even in trace amounts, of a substance, which may or may not be toxic, capable of binding to this receptor.

The present invention also extends to any ligand for a receptor of interest, identified using the method claimed.

The examples and figures submitted hereinafter are presented by way of nonlimiting illustration of the present invention.

FIGURES

Figures 1:

Figure 1a: Visualization by confocal microscopy of the neurotensin type 1 receptor coupled to the fluorescent protein EGFP (NTR1-EGFP) at the surface of CHO cells incubated with buffer alone or with a low concentration (1 nM) of rat or frog neurotensin, which does not induce internalization.

Figure 1b: Visualization of the internalization of the NTR1-EGFP receptor, characterized by the formation of numerous fluorescent cytoplasmic vesicles with a diameter of 0.6 μm in CHO cells incubated with neurotensin concentrations of 10 and 100 nM.

Figures 2: Visualization of the internalization of the NTR1-EGFP receptor with or without acid washing, in the presence of: 10 nM of neurotensin (figure 2a) or of a purified fraction of frog brain extract (figure 2b). The conditions are the same as in figure 1 and also comprise an acid wash of the endogenous ligand still bound to the surface of the cells at the end of the loading period.

Figure 3: Internalization of the NTR1-EGFP receptor in the presence of 10 prepurified fractions of a frog brain extract.

Figure 4: Radioimmunological assay of the prepurified fractions of a frog brain extract using an antibody directed against the conserved region of neurotensin.

Materials and methods

1) Construction of the NTR1-EGFP receptor

The entire coding sequence of the NT1 receptor (K. Tanaka, M. Masu and S. Nakanishi (1990) Structure and functional expression of the cloned rat neurotensin receptor. Neuron 4: 847-54) was amplified by PCR using two primers directed against the 5′ and 3′ ends of the coding sequence of this cDNA and also containing HindIII or BamH1 restriction sites, 5′-CTT AAG CTT ATG CAC CTC AAC AGC TCC GTG-3′ (SEQ ID No. 1) and 5′-TTT GGA TCC GCG TAC AGG GTC TCC CGG GT-3′ (SEQ ID No. 2). After digestion with the HindIII and BamH1 enzymes and purification, the amplified sequence was inserted into the pEGFP-N1 expression vector (Clontech) at the HindIII and BamH1 sites. The construct was verified on an AbiPrism 377 automatic sequencer (Perkin-Elmer) using fluorescent ddNTPs.

2) Stable transfection in CHO cells

The CHO-K1 cells (American Type Culture Collection: ATCC) were cultured in a humid atmosphere at 5% CO_2 in F12 medium supplemented with 7.5% of fetal calf serum, 1 mM of glutamine, 100 units/ml of penicillin and 100 units/ml of streptomycin (Boehringer Mannheim). In order to establish the stable line expressing this receptor, the CHO-K1 cells (approximately 2.6 × 106 cells) were transfected with 8 μg of plasmid using cationic liposomes (Dosper, Boehringer). The transfected cells are selected for their resistance to geneticin. The clones obtained were sorted using flow cytometry, which takes into account the intensity of the fluorescence of each cell. A second selection was

carried out using a fluorescent microscope, by observing the level of expression of the fluorescent NTR1-EGFP receptor located at the cell membrane surface for each of the clones.

3) Prepurification of the frog brain extract

- Collection of tissues

2541 male green frog brains (species Rana ridibunda), corresponding to a fresh tissue weight of 215 g, were collected in the laboratory from freshly sacrificed animals. The brains were frozen on dry ice immediately after having been removed, and stored at -80°C.

- Extraction of tissues

The brains were immersed in boiling 0.5 M acetic acid (2 liters) for 15 min and then homogenized in a mixer. The homogenate was centrifuged at 4000 × g for 30 min at 4°C. The supernatant was then prefiltered over an assembly of 10 C18 Sep-Pak columns (Waters Associates, Milford, MA) mounted in series, at a flow rate of 2 ml/min. The material bound to the Sep-Pak columns was eluted with 20 ml of a 70% acetonitrile solution. This operation was repeated twice.

- Purification of the frog brain extract by semipreparative HPLC

The material eluted from the Sep-Pak columns was partially evaporated in order to remove the acetonitrile, and then centrifuged at 13 000 × g for 5 min. The supernatant was removed and divided in 2. Each of the 2 pools was then injected onto a semipreparative Vydac C18 218TP1010 column (1 × 25 cm) (The Separations Group, Hesperia, CA) equilibrated with a solution of water/trifluoroacetic acid (99.9:0.1; vol:vol) at a flow rate of 2 ml/min. The peptide

material bound to the matrix was eluted using an acetonitrile gradient rising from 14 to 42% in 40 min at a flow rate of 2 ml/min, and then increasing from 42 to 56% for 60 min at a flow rate of 1 ml/min. The eluate was collected in 1 min fractions and the absorbance was measured at 215 and 280 nm. The elution fractions were stored at -20°C. Before the internalization experiments, each of the fractions was diluted with water, partially evaporated in order to remove the acetonitrile and made up to a final volume of 50 µl.

4) Internalization

The cells are distributed (50% confluency, 20 000 cells per well) and then cultured overnight on multi-well slides (LabTek, Nunc) pretreated with polyallylamine (0.1 mg/ml, Aldrich). The incubation volume (except for the loading period) and washing volume is 250 µl per well on these slides. 90 min before the start of the experiment, the cell medium is exchanged for a medium supplemented with cycloheximide (70 µM, Sigma). The cells are then preincubated for 15 min on ice in cold Earle's buffer (pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 3.6 mM $MgCl_2$, 0.1% bovine serum albumin, 0.01% glucose and 0.8 mM 1,10-phenanthroline). The cells are then incubated for 30 min with the ligand diluted in 50 µl of Earle's buffer at 4°C (loading period). At this stage, a batch of cells is subjected to a hypertonic acid wash (0.2 M of acetic acid and 0.5 mM of NaCl in Earle's buffer, pH 4, for 2 min at 4°C) in order to dissociate the ligand from its surface receptors. The internalization is caused by replacing the medium with Earle's buffer at 37°C and incubating the slides at 37°C for 20 to 30 min (chase period). At the end of incubation, the cells are rinsed with cold Earle's buffer, fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer at pH 7.4, rinsed

again in cold Earle's buffer and then mounted using Vectashield (Vector).

5) Confocal microscopy

The cells are examined with a Leica TCS NT confocal microscope in a configuration with an inverted microscope (Leica DM IRBE) equipped with an argon/krypton laser with excitation and emission filters of 488 and 530-600 nm, respectively. 1024-1024 pixel images of individual cells are obtained using a 63x oil-immersion objective.

6) Radioimmunological assay for neurotensin on prepurified fractions of a frog brain extract

A 5 µl volume of each fraction collected at the semipreparation HPLC outlet is subjected to a radioimmunological assay for neurotensin. The assay for neurotensin was carried out using an antibody directed against the C-terminal fragment of pig neurotensin (Marcos et al., Peptides 1996, 17: 139-146).

The antibody is used at a final dilution of 1:50 000, and the sensitivity of the assay is 10 pg. The radioimmunological assay is carried out at 4°C in a 0.02 M veronal buffer (pH 8.6) containing 4% of bovine serum albumin and 7000 cpm of (3-[125I] iodotyrosyl) neurotensin (Amersham, Buckinghamshire, UK). The samples and the antibody were incubated at 4°C for 48 h. The separation of the tracer fraction bound to the antibody was carried out by precipitation by adding to each sample a solution of γ-globulins (1% in 0.02 M veronal buffer) and a solution of polyethylene glycol (20% in a 0.02 M veronal buffer containing 0.1% of bovine serum albumin and 0.1% of triton X-100). After a 20 min incubation at room temperature, the samples are centrifuged (3000 × g, 30 min) and the pellets counted in a gamma counter.

EXAMPLE 1: Internalization of the NTR1-EGFP receptor in the presence of frog or rat neurotensin

1) Characterization of the NTR1-EGFP receptor

The NTR1-EGFP receptor is stably expressed in the CHO cell line and its binding and intracellular signal transmission properties were determined. The affinity of neurotensin proved to be of the same order (0.3 nM) for the NTR1-EGFP receptor and the wild-type NT1 receptor. Similarly, the labeled receptor leads to the production of inositol phosphates with an EC50 (1 nM) which is identical to that obtained for the wild-type NT1 receptor (results not shown).

2) Internalization of the NTR1-EGFP receptor in the presence of frog or rat neurotensin

The cells incubated with buffer alone or with low concentrations (concentrations less than 1 nM) of rat or frog neurotensin exhibit marked fluorescence of the NTR1-EGFP receptor, located at the cell membrane (fig. 1a).

The incubation of the CHO-NTR1-EGFP cells with increasing concentrations of neurotensin (range beginning at 10 nM) leads to internalization of the NTR1-EGFP receptors, indicated by a decrease in the fluorescent membrane labeling and by the formation of numerous fluorescent intracytoplasmic vesicles with a diameter of 0.6 μm (fig. 1b). This type of labeling is not detected when the ligand is dissociated from the surface receptors beforehand subsequent to an acid wash carried out at the end of the loading period (fig. 2a). These results indicate that the internalization observed is the result of the binding of the neurotensin to the membrane receptors during the loading period.

EXAMPLE 2: Internalization of the NTR1-EGFP receptor in the presence of prepurified fractions of a frog brain extract

In a first series of experiments, 0.5 µl of each of the 120 elution fractions of a frog brain extract (prepurified on a semipreparative column) were pooled in 10s, producing 12 pools of 10 fractions, each made up to 50 µl with Earle's buffer. Only pool 2 containing fractions 11 to 20 leads to the internalization of the NTR1-EGFP receptor.

In a second series of experiments, the fractions of pool 2, i.e. fractions 11 to 20 (0.5 µl of volume of original fraction diluted to 50 µl with Earle's buffer), were tested individually. Only fractions 15, 16, 17 and 18 lead to the internalization of the NTR1-EGFP receptor (fig. 3). This internalization is not detected if the cells are subjected to an acid wash at the end of the loading period (fig. 2b), indicating that the internalization observed is the result of the binding of a specific ligand to the NTR1-EGFP receptors during the loading period.

EXAMPLE 3: Radioimmunological assay for neurotensin on the prepurified fractions of a frog brain extract

The radioimmunological assay of the prepurified fractions of a frog brain extract using an antibody directed against the conserved region of neurotensin shows that the immunoreactive material is exclusively contained in fractions 15, 16, 17 and 18 (fig. 4). The apparent total amount of neurotensin measured in the elution fractions from the semipreparative HPLC is 894 ng in 16 ml, which corresponds to a value of 352 pg of peptide per frog brain. Consequently, the material causing the internalization of the NTR1-EGFP receptor,

contained in fractions 15, 16, 17 and 18, corresponds to the endogenous neurotensin of the frog brain.

In conclusion, the internalization method described above is a direct, simple, specific and reliable means for detecting, based on the observation of a single cell, an amount of neurotensin as small as 500 fmol in 50 μl. It appears that this measurement can be carried out both on a pure solution of neurotensin and on a fraction of tissue extract containing not only the neurotensin but also a large number of other neuropeptides (the minimum number estimated per fraction being 50 peptides), with a similar sensitivity since, according to the RIA assay, it is possible to detect approximately 250 fmol.